EVALUATION OF FATIGUE RESISTANCE IN ALASKAN SLED DOGS THROUGH EXERCISE INDUCED MYOCYTE GENE EXPRESSION

A Thesis

by

NATACHA MARIA SALAZAR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Theresa Fossum
Committee Members,	Michael Davis
	Vicky Haines
	Ivan Ivanov
Head of Department,	Sandee Hartsfield

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ABSTRACT

Evaluation of Fatigue Resistance in Alaskan Sled Dogs Through Exercise Induced Myocyte Gene Expression. (December 2010)

Natacha Maria Salazar, DVM, Universidad Nacional Experimental Francisco de Miranda

Chair of Advisory Committee: Dr. Theresa Fossum

The physiological responses to exercise depend on intensity, duration, and type of exercise. The muscles in the body have complex regulation responses in order to create a certain resistance and adaptation to the exercise demands without fatigue. In the following study, we used the model of Alaskan sled dogs in order to analyze changes in gene expression within muscle tissue. Gene expression allows us to look more in depth into temporal or long term biological changes that take place in order for the muscle to adapt and maintain homeostasis. Eight dogs were used for the study; four biopsies from the femoris biceps were taken from each at different time points. Time point 1 (Tp1) untrained dogs, time point 2 (Tp2) after mid training, time point 3 (Tp3) fully trained and time point 4 (Tp4) were taken after dogs had completed a 400 mile run in 4 consecutive days.

Time point one was used as a control ratio for the other three time points for analysis one, for the second analysis Tp1 was eliminated as a control. Analysis, one compared Tp2-Tp3 and Tp3-Tp4; the subsequent analysis looked at Tp1-Tp3. For Mid trained animals compared to fully trained, we looked at a total of 25 differentially expressed genes, for fully trained compared against acute exercise performance, we looked at total of 52 differentially expressed genes (based on a ≤ 0.01 p-value and fold change of ≥ 3), and untrained was compared to fully trained where we looked at a total of 26 differentially expressed genes. Known transcriptional regulators

were mapped from these differentially expressed genes, such as exocyst complex, lysyl oxidase, protein tyrosine phosphatase, protein kinase C, creatine kinase, HSP40, cytochrome P450, ACSL6 gene responsible for Acyl-CoA synthesis, myosin chain, ATP binding, and ubiquitin, among others. These transcripts were linked to important biological pathways, and functional analysis of these pathways demonstrated that changes found in gene expression are responsible for muscle tissue remodeling, energy storage and metabolism changes, cardiovascular enhancement, and activation of elements that regulate metabolism via the nervous system. The following study of transcriptional regulation mechanisms helped identify specific responses to exercise stimuli in the organism that allow the athletes to adapt to the demands of exercise.

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I would like to thank my parents for always believing in me and pushing me to strive at all my goals.

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Thanks to my wonderful husband, Russell Montalvo, for his support and love through these last months of school, and for motivating me to endure long nights without sleep for my research.

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1. INTRODUCTION

Endurance training has been shown to create physiological changes at the muscle level that give the individual the ability to engage in long bouts of exercise, sustaining a certain workload and achieving high power output for longer distances or times. However, this ability may also be a consequence of genetic endowment. Muscle responds by increasing its production of energy through ATP to meet the demands of the exercise. It can also hypertrophy in order to adapt to long term work requirements. Exercise muscle cells are exposed to 4 different important stressors: mechanical load, metabolic disturbances, neuronal activation and hormonal alterations. At a simplistic level these stressors work at the skeletal and smooth muscle level causing improved cardiovascular functions as well as changes in substrate oxidation towards lipids rather than carbohydrates [1, 2].

Skeletal muscle responses to training are regarded in gene expression changes within the muscle. The molecular mechanisms responsible for muscle remodeling, resistance, and adaptation to damage after bouts of exercise is a field of research starting to be unraveled through accumulating evidence that gene-specific transcriptional activation is involved [3]. These transcriptional changes are dependent of the type, duration and intensity of the exercise [4]. It is believed that at some level, the event of transcriptional activation is associated with reestablishing homeostasis in skeletal muscle, as well as contributing to the skeletal muscle adaptations that occur in response to exercise training [5]. There are many known myogenic and metabolic genes that play important roles in cell regulation, yet there are many that have not yet been identified. According to Yang [6], there is strong evidence to support that gene regulation at the myogenic and metabolic levels occurs after acute bouts of exercise. The identification of

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these genes comes as a definite importance in transcriptome profiling of skeletal muscle function. One of the most known enhanced expression resulting from muscle adaptation to training, is the mitochondrial genes and genes involved in oxidative stress metabolisms [1, 7]. But despite extensive research in the area of exercise physiology both in humans and animals, time dependent changes in skeletal muscle gene expression are not well defined. The study of gene expression in skeletal muscle tissue is a useful way to explore aspects of exercise physiology and changes that may take place during muscle adaptation to different regimens of exercise training. It is thus important to characterize differentiation of gene expression responses to different training intensities using an important well known model.

Alaskan sled dogs serve as a valuable model due to their ability to withstand sustained submaximal exercise for long periods of time [8]. These sled dogs used in competitions undergo rigorous training and extended exercise bouts, they are considered exceptional athletes. Other studies done using other models such as trained human athletes or rodents have not shown nearly the same resistance to sustained exercise [9, 10]. Skeletal muscle glycogenolisis is greatly reduced in these athletes, they undergo consecutive days of prolonged submaximal exercise in favor of the use of alternative energy sources [11]. This phenomenon occurs rapidly with muscle glycogen utilization virtually ceasing by the fourth day of exercise, and is likely related to a combination of innate ability (genetic endowment), dietary composition and intensive endurance training. Dr. Davis [8] has demonstrated gradual replenishment of muscle glycogen over five days of repeated prolonged exercise despite a relatively limited carbohydrate intake. This phenomenon appears to result from dramatic attenuation of glycogen utilization during repeated bouts of prolonged low intensity exercise in fit sled dogs consuming a relatively high fat diet.

Microarray technology is a sensitive and powerful tool for identifying changes in gene expression with relatively small amounts of tissues or cells. This method provides a way to

identify novel types of genes that respond to different stimuli and enhances research exploring of transcriptional contributions to cellular events [5, 12]. RNA is responsible for fine regulation of gene expression. Changes in these RNA may alter the cellular phenotype of the muscle tissue during delicate processes known as post-transcriptional gene regulation. Muscle fibers regulate abundance of protein through these processes of transcription and translational mechanisms; mRNA synthesis is then regulated by the rate of these mechanisms over the gene encoding.

The purpose of the present research was to determine important molecular adaptations of skeletal muscle tissue to rigorous exercise and acute exercise by analyzing gene expression changes. Gene expression analysis allows detailed evaluation of the transcriptional changes caused by training interventions and long bouts of exercise, more so than functional testing and structural analysis. Using the sled dog model we were able to study time-dependent changes and responses to different training levels. We hypothesized that gene expression patterns may help in determining new and important regulatory elements that take place allowing the athlete to adapt and resist long bouts of exercise without fatigue. A second hypothesis was that the regulatory elements that take place in order to allow conditioning of the skeletal muscle to exercise may change in sight of acute exercise demands.

2. METHODS

Animals and related procedures. Approved by the Oklahoma State University Institutional Animal Care and Use Committee. Informed consent was obtained by the owners before initiation of study. Ten Alaskan huskies were used for this study. All dogs came from the same kennel, they were fed the same food (commercial kibble) and at the same time each day during the whole study. Their ages ranged from 2.5 to 7 years (4.7 mean ± SD), they were evenly mixed females and males, and their weight ranged from 22 to a max of 28kg (average weight was 25kg). Exercise training and acute exercise consisted of running as a team (side by side) as accustomed for sled racing over packed snow.

Each dog was examined at 4 different time points related to training and development of sub maximal exercise. The four time points were pre-training, mid-training, fully trained and post exercise. These 4 time points are as described: pre-training; defined as extended rest of at least 4 months with no forced exercise. Mid-training, defined as able to complete at least 10miles/day of exercise on consecutive days without fatigue. Fully trained, defines as able to complete at least 50miles/day of exercise on consecutive days without fatigue. Post exercise, defined as having completed a 400 mile exercise challenge in 4 days without fatigue. This exercise challenge has shown to induce a unique, fatigue – resistant phenotype in which there is minimal reliance on intramuscular substrate to support the exercise.

For collection of muscle biopsies a 2cm x 2cm site over the middle of the biceps femoris muscle had the guard hairs taped back and the underlying hair clipped. Dogs were anesthetized with an initial intravenous bolus of propofol (5mg/kg), and sufficient anesthesia maintained with additional intravenous boluses of propofol. After hair was clipped the area was scrubbed using

clorhexidine scrub, a fenestrated sterile paper drape was placed over the clipped site. A stab incision was made in the skin using a #15 scalpel blade and a commercial biopsy needle (12 gauge) inserted into the belly of the muscle with each biopsy, and a maximum of 3 biopsies per leg were performed. Each biopsy was immediately placed in a cryovial, snap frozen in liquid nitrogen, and stored at -80ºC until analysis. The 4 time points for the biopsies were: September-07-2007, November-2-2007, January-6-2008, and January-12-2008 respectively. The last biopsy was taken 6 days after the previous; this corresponds to 2 days of recovery from the previous muscle biopsy then 4 days for covering the 400miles.

RNA extraction. Total RNA was extracted using procedures established by Dr. Peter Hoyt (2009), Head of the Microarray Core Facility at Oklahoma State University. Quiagen purification columns were used for isolation of RNA and digestion of genomic DNA following manufacturer's instructions. All samples were labeled with the name of the dog, followed by the date the sample was taken; labels were placed on the microtubes at all times to keep track of samples and stage of performance.

Quantification and quality testing of RNA extraction. Performed using NanoDrop (ND-1000) testing technology provided at the Oklahoma State University Core Facility. This machine uses 1µl of the solution containing the eluted RNA for each sample and provides measurements of RNA quantity in ng per µl and quality of RNA. Gel electrophoresis was used to provide further proof of the quality of RNA**,** absence of DNA or contaminating agents were verified through this method for all samples.

Amplification of RNA for labeling. For this step we used a TargetAmp Aminoallyl-aRNA Amplification Kit by Epicentre Biotechnologies and aRNA purification to remove unincorporated nucleotides from our amplified aRNA using MiniElute quiagen columns**.** This procedure further purified the RNA obtained in the previous steps. Amplification was performed

adding an aRNA tail sequence. This procedure was necessary for obtaining a larger quantity of purified RNA for each sample in order to perform the labeling and hybridization.

Labeling of amplified aRNA for each sample using 4 alexa dyes. One color dye was used for each time point. Ten µg (10µg) of amplified RNA for each sample-timepoint was placed in a vacuum dry desiccator. Once it was vacuumed dry, 5µl of sodium carbonate buffer were added to each sample and mixed periodically by flicking during 20 minutes at room temperature. Four dyes were used to label the samples according to timepoint: Alexa dye 488 was used on all samples 9707, Alexa dye 532 was used for all samples 11207, Alexa dye 594 was used on all samples 1608, Alexa dye 647 was used for all samples 11208, each corresponding to a sample obtained at a specific stage of exercise. A total of 5µl of the appropriate dye was added to each sample. The resulting solution was incubated for 2 hours at room temperature in complete darkness. To make sure that the solutions were not exposed to light each micro tube was covered with aluminum foil and placed in a light proof container for the incubation period, and all the handling of the samples thereafter.

After incubation each sample was subjected to the aRNA purification protocol using the MiniElute quiagen columns. This last procedure eliminated any excess dye adhering to the RNA. Final samples were placed in a fresh micro tube (previously labeled with the corresponding sample name), once more protected from light, and tested to verify quality and quantity of labeled RNA. Once they were tested, they were stored at -80˚C.

Hybridization of samples. Samples were thawed (in complete darkness) and then hybridized into the array slides. Hybridization of samples was performed following the Agilent Microarray-Based Gene Expression Analysis protocol (Quick Amp Labeling), and the Agilent microarray hybridization chamber user guide for hybridization of the slides. The hybridization process took 12 hours per array, each array was placed in a rotator rack within a hybridization

oven with constant rotation control and programmed at a temperature of 65ºC. The rotation allows the array to be hybridized properly across the chamber. After the 12 hours arrays were washed with wash solution buffer 1, sandwiched slides completely submerged are pried apart, the slide with the barcode is passed to a second wash with the same solution (being careful to minimize exposure of slide to air). (All procedures followed Agilent microarray hybridization chamber user guide version 2.0, August 2006).

Scanning of the arrays. The arrays were scanned with an Agilent multi color scanner. This scanner has the ability to perform a scan of the four-color arrays separately and also compare signal wavelength between the different colors to optimize signal quality and reduce background noise of the arrays. This procedure was done at the Oklahoma State University Microarray Facility using their equipment.

Analysis of the microarray data. Using GenePix-Pro to obtain the scanned data, ratios were set up to compare the different wavelength of each color over a control factor. Tp1 was colored with Alexa dye 488, this became the control factor. GenePix-Pro normalized all the data for each set of log-base ratio using 488 spots as the denominator to a median of "1". Using dog 1 through dog 8 the software used all the 647/488 ratios for Tp4, 594/488 ratios for Tp3, and 532/488 ratios for Tp2, then data was combined using average mean for each time point. Data collected from the arrays were obtained in Excel .GPR (GenePix Results) format for analysis. Data files were then processed through GPAP3.2 to verify that variability in gene expression between dogs in a single time point was low.

To analyze the data obtained from GenePixPro consisting of gene annotation, log ratios, and flagged spots, data corresponding to different time points were uploaded into MeV (MultiExperiment Viewer) [13]. Once data were uploaded we proceeded to adjust it using the GenePix flag filter to eliminate all control spots (negative and positive controls, dark corners and

GE_bright corners). Once this step was performed data for each individual time point were adjusted using mean center sample. This procedure was done in order to center the data for each spot into a standard normal distribution for all dogs in a single timepoint. Then data were normalized by intensity to a Gaussian distribution. The result of this adjustment was then set as the main data source for continued analysis. A second data adjustment were performed using variance filters, this procedure ranks the genes based on their standard deviation using a highest percentage of SD genes across all samples. Filter was placed at 20%, giving a total of 8056 most variable genes for both studies. These procedures were performed comparing time point 2 (mid-training) with time point 3 (fully trained), and time point 3 to time point 4(after acute exercise performance). A TTest was applied for each experiment using alpha of 0.05 and 0.01 to determine most significant genes that underwent expression changes among the experiments and K-means clustering was applied to the results. The resulting transcripts were than introduced into The Ingenuity Pathway Analysis (IPA) system to determine biological function analysis and for pathway networks (Ingenuity® Systems, www.ingenuity.com).

In order to study time point one (untrained animals) with time point 3 (fully trained), a second analysis was performed using the raw data without the 488(Tp1) control ratio. We performed this study to determine gene expression changes between the two completely different physiological stages (untrained animals to fully trained animals). All analysis for these two time points was performed following the same procedures described above, and the same analysis software (MeV and IPA).

3. RESULTS

Data samples from two out of the ten dogs used for this study were eliminated due to low RNA yields obtained during the process of RNA extraction for these two animals. Minimal non-statistically significant variation in gene expression among subjects when looking at the same time point were ascertained as biological variability due to differences such as sex, breed weight and age of the dogs. This was tested through data analysis performed by GPAP3.2 across the same data point for all animals, the standard deviation (SD) of log base 2 expression patterns between the dogs at Tp2 was of 1.7, for Tp3 was of 1.5 and for Tp4 was of 1.45. This demonstrated that gene expression patterns taken from a single time point did not differ greatly compared to that of different time points. This means that physiological variability between test subjects did not affect gene expression patterns observed for single time points.

To determine relevant differentially expressed genes between mid trained dogs and fully trained dogs, as well as between fully trained dogs and those that completed the acute exercise endurance, we performed a T-test with a 0.01 P-value using the data set of genes that had the highest percentage of SD. Using this criteria we obtained 24, and 52 genes differentially expressed for the Tp2-Tp3 exercise training time points and between Tp3-Tp4, respectively. Results also showed that only 1 gene overlapped between studies. Curiously this gene transcript (type I cytoskeletal, cytokeratin 16) showed different regulation in the two studies, being up regulated in the Tp4 study and down regulated in Tp2-Tp3.

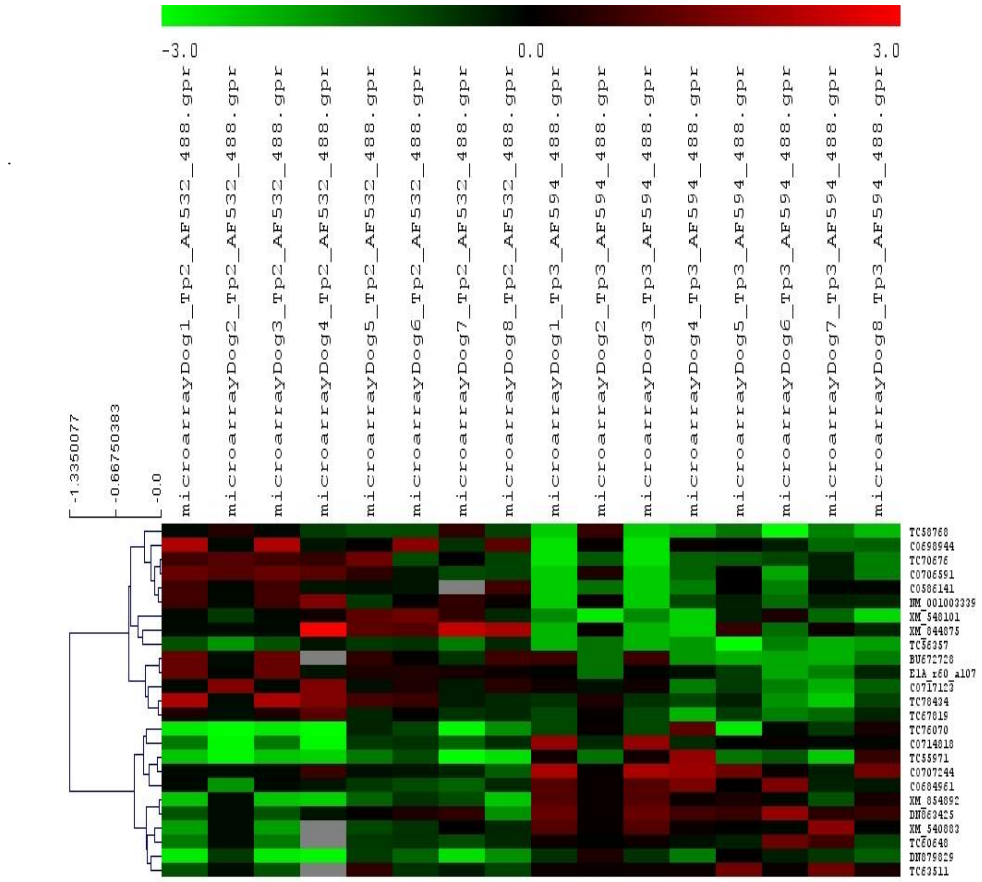

Fig 1. Gene expression patterns with KMC clusters of significant genes found between time points 2 and 3 post exercise. Transcripts chosen as representing a significant differential expression change between the two time points had an absolute t-value ≥ 3 and a p-value ≤ 0.01. The KMC clustering showed all differentially expressed genes separated by regulation and fold change. Different time points for each dog are represented here as well as GeneID

Clustering of the differentially expressed genes showed the temporal patterns of gene expression between the two separate results for the two studies performed between time points (Figs. 1 and 2). Clustering also helped separate the transcripts by their different regulations.

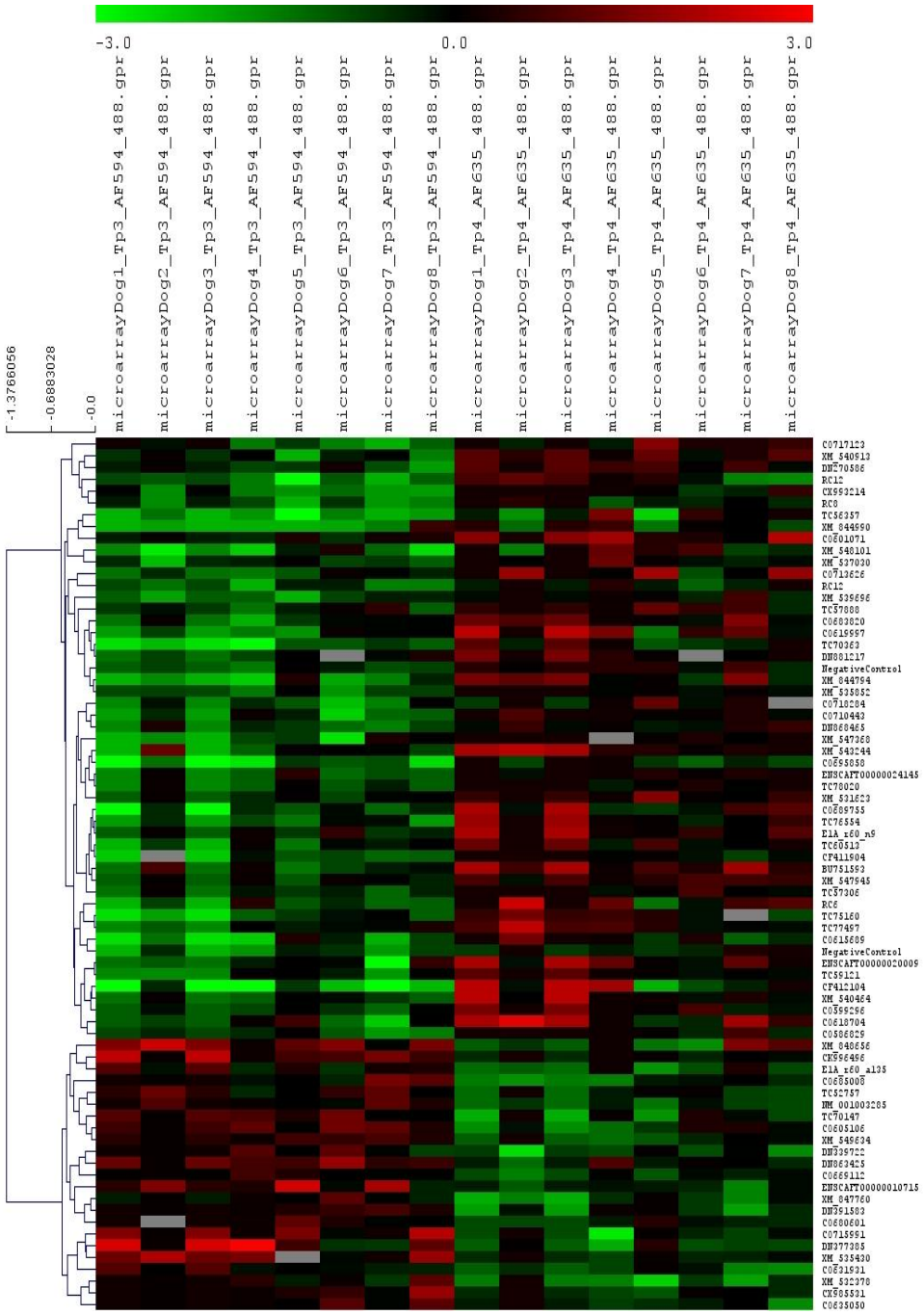

Fig 2. Gene expression patterns with KMC clusters of significant genes found between time points 3 and 4 post exercise. Transcripts chosen as representing a significant differential expression change between the two time points had an absolute t-value ≥ 3 and a p-value ≤ 0.01. The KMC clustering showed all differentially expressed genes separated by regulation and fold change. Different time points for each dog are represented here as well as GeneID.

IPA biological functions helped us find the specific transcripts for each of the gene IDs and their biological significance in pathway analysis. Out of the 24 genes found to be significant in the Tp2-Tp3 study IPA mapped 5 genes (table 1) as relevant for a biological pathway, unfortunately many of the genes found to be differentially expressed have no current annotations, and therefore could not be studied further. In the case of the 52 significant genes between Tp3-Tp4 IPA mapped 13 relevant transcripts (table 1). The changes in expression observed between the time points for these mapped genes gives us important insight for characterizing muscle transcriptional response to training and acute exercise.

Table 1. Genes found to have the greatest altered gene expression and relevant biological mapping by IPA.

Gene	*Transcript*	*Tp2-3*	*Tp4-5*
EXOC1	exocyst complex component 1	+3.7	NS
CCDC88B	coiled-coil domain containing 88B	+3.4	NS
KRT14	keratin 14	-3.8	+3.1
ACSL6	acyl-CoA synthetase long-chain family member 6	-3.1	NS
CYP2E1	cytochrome P450, family 2, subf E, polypeptide 1	-3.4	NS
LOXL2	lysyl oxidase-like 2	NS	+3.05
EPHA6	EPH receptor A6	NS	+4.2
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	NS	+3.2
CAMSAP1L1	calmodulin regulated spectrin-associated protein 1-like	NS	+3.7
PACSIN1	protein kinase C and casein kinase substrate in neurons	NS	+3.1
PTPN21	protein tyrosine phosphatase, non-receptor type 21	NS	+3.9
CHIA	chitinase, acidic	NS	+3
ZBBX	zinc finger, B-box domain containing	NS	+4.8
DNAJC5G	DnaJ (Hsp40) homolog, subfamily C, member 5 gamma	NS	-3.2
FSIP1	fibrous sheath interacting protein 1	NS	-4.4
RLBP1	retinaldehyde binding protein 1	NS	-4.6
PDCL2	phosducin-like 2	NS	-3.8

IPA linked the genes found to be differentially expressed to specific biological pathways involved in the regulatory changes taking place in the muscle tissue, metabolism and cardiovascular system during exercise. The pathway for the coiled-coil domain containing 88B is involve in ATP binding, enzyme, metallopeptidase, mitogen-activated protein kinase p38 binding, protein binding, ribosomal protein S6 kinase, and zinc ion binding. Another pathway found to be important for transcript in Tp2-Tp3 comparison was the exocyst complex component 1 (fig 3). Using pathway builder in IPA we were also able to observe interaction and functions of each of the genes differentially expressed for both studies (Fig 4,5).

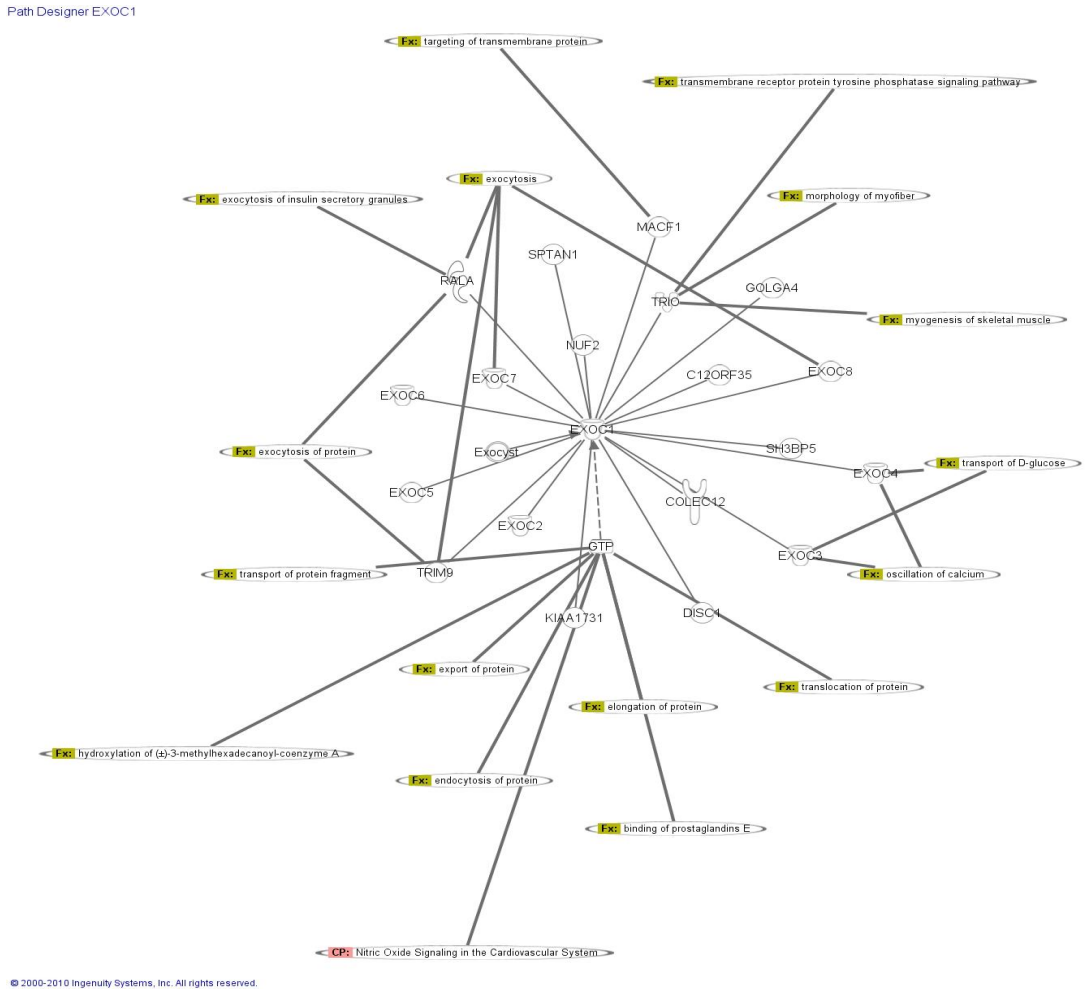

Fig 3. Pathway shows the interactions between EXOC1 and other transporter genes, as well as important functions regulated by this pathway.

In our subsequent analysis performed of Tp1 and Tp3 we found 1792 differentially expressed genes. The full set of genes (1792) were introduced into the IPA software which mapped 860 genes with specific regulatory functions. The most important regulatory functions found to be affected by these transcripts were: lipid metabolism (57 transcripts), tissue development (59 transcripts), muscular system development (39 transcripts), Energy production (18 transcripts), carbohydrate metabolism (35 transcripts), and amino acid metabolism (24 transcripts), (Supplementary Data). In order to adjust the data and look more in depth into the most important sets of genes, we filtered out all genes that had a fold change ≤ 3, and were subsequently left with 51 down regulated genes and 9 up-regulated genes. Than we introduced our genes that possessed more than 3 fold change throughout the Tp1-Tp3 study into IPA which mapped 5 out of the 9 up-regulated genes and 30 of our down-regulated genes (Table 2).

Table 2. Differentially expressed genes found between Tp1 and Tp2 and their relevant biological functions as shown by IPA.

Gene	*Fold change*	*Function*
PDHB	-3.2	pyruvate dehydrogenase (lipoamide) beta
MYL2*	-3.7	myosin, light chain 2, regulatory, cardiac, slow
GSTK1	-3.4	glutathione S-transferase kappa 1
EEF1D*	-3.5	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)
ATP5A1	-3.6	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle
SLC25A3*	-3.8	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3
MYH7*	-3.4	myosin, heavy chain 7, cardiac muscle, beta
TNNT1	-7.5	troponin T type 1 (skeletal, slow)
PGM1	-3.5	phosphoglucomutase 1
FBP2	-4.9	fructose-1,6-bisphosphatase 2
ALG13	-3.1	asparagine-linked glycosylation 13 homolog
GOT1	-3.6	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)

Table 2. Continued'

Gene	*Fold change*	*Function*
PGK1	-3.6	phosphoglycerate kinase 1
MB	-4.4	Myoglobin
ATP5O	-3.3	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit
SOD2	-3.2	superoxide dismutase 2, mitochondrial
CA3	-3.9	carbonic anhydrase III, muscle specific
BRP44	-3.3	brain protein 44
SYNPO2	-1.4	synaptopodin 2
CKMT2	-10.8	creatine kinase, mitochondrial 2 (sarcomeric)
GAPDH	-4.5	glyceraldehyde-3-phosphate dehydrogenase
MYL2	+3.7	myosin, light chain 2, regulatory, cardiac, slow
GAPDH	+3.1	glyceraldehyde-3-phosphate dehydrogenase
VDAC2	+1.6	voltage-dependent anion channel 2
UQCRFS1	+1.4	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
CHCHD10	+1.7	coiled-coil-helix-coiled-coil-helix domain containing 10

Important functions affected by these genes energy metabolism such as catabolism of ATP, accumulation of pyruvic acid, biosynthesis of ADP, biosynthesis of Acetyl-CoA, gluconeogenesis, glycolysis, metabolism of carbohydrates and amino acids, utilization of D-glucose, and free radical scavenging such as conversion of superoxide and reactive oxygen species.

4. DISCUSSION

Exercise training and acute exercise causes skeletal muscle responses and remodeling which aid the athlete in adapting to different bouts of exercise, these adaptations tend to be short or long-termed depending on the requirements and/or genetics of the individual. The current analysis of gene expression in a group of Alaskan sled dogs showed significant information as to pathways and functions altered in the muscle in order to allow these athletes to perform extraordinarily. Though many of the changes that take place to allow an athlete to adapt to strenuous exercise are incompletely understood, the results of this study help elucidate some of the more important changes that take place at a metabolic level.

The EXOC1 gene (fig 3) is part of an exocyst complex which confers efficient cellular migration through its ability to control activation of certain subpopulations of MAPKinases [14]. This transcript was found to be up-regulated between Tp2 and Tp3. Multiple myosin II molecules generate force in skeletal muscle through mechanisms fuelled by the energy released from ATP hydrolysis. Power is generated by the stroke of release of phosphate from the myosin molecule after the ATP hydrolysis while myosin is tightly bound to actin. The exocyst complex acts over the regulation of vesicles transported by the type V myosin along polarized cables of actin to sites of cell surface growth [15]. It is of no surprise that this transcript is altered in order to allow a greater force to be carried out through the skeletal muscle for the exercise requirements that these athletes must endure.

Between time point 2 and time point three we observed a 3 fold change down regulation of the ACSL6 gene. This gene is responsible for the activation of long chain fatty acids in erythrocytes, fatty acid degradation, prospholipid remodeling and production of long Acyl-CoA

esters that are in charge of regulating certain physiological functions [16]. Fatty acid transport proteins are highly expressed in skeletal muscle and modulate the fatty acid metabolism. Alteration of these metabolisms are known to cause insulin resistance in the muscle.

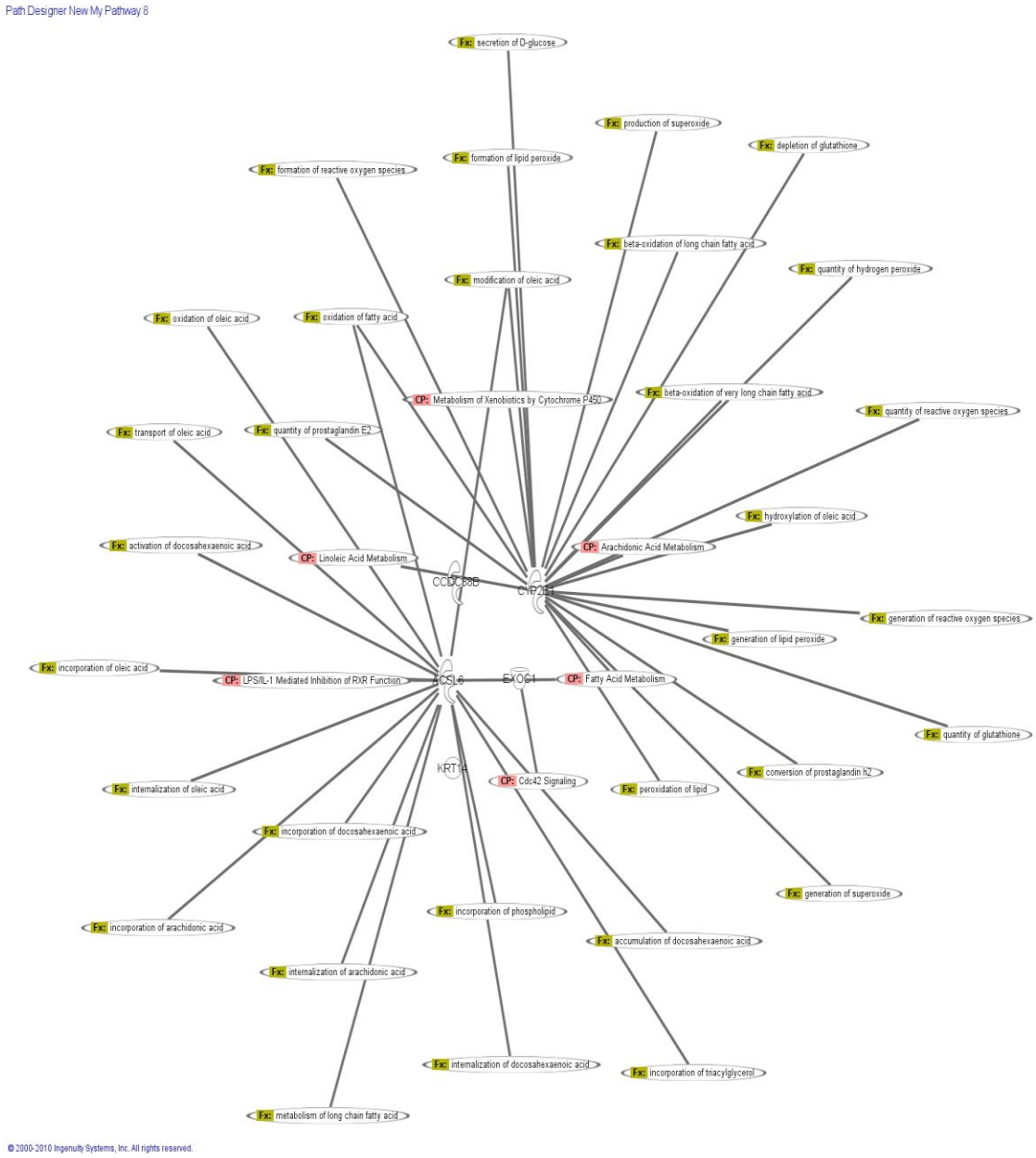

Fig 4. Pathway for interaction of the 5 genes differentially expressed between Tp2-Tp3 as well as their know functions.

The Overexpression of mouse fatty acid transport protein (FATP1) in cultured fibroblasts resulted in a marked increase in long-chain fatty acid uptake. However, the inactivation of Excessive fatty acid derived metabolites can also blunt insulin signaling activity [17].

CSL6 gene is also known to form part of the canonical pathway for LPS/IL1 mediated inhibition of the RXR function (Fig 4). The RXR forms part of the family of nuclear receptors which are involved in mediating the effects of retinoic acid (RA). This receptor forms heterodimers with the retinoic acid, thyroid hormone, and vitamin D receptors, increasing both DNA binding and transcriptional function on their respective response elements. Change of transcript level do not necessarily correlate to changes in the protein levels. However, down-regulation of the ACSL6 gene could be a metabolic response that enables fatty acid metabolism changes required for alternate energy production in the muscle tissue needed for endurance.

CVP2E1 is the cytochrome P450 transcript. This gene is more commonly known for its ability to oxidize xenobiotic compounds, endogenous fatty acids, including lipids associated with mechanisms such as that of arachidonic acid. The cytochrome P450 binds to mid length fatty acids which then catalyzes hydroxylation of saturated fatty acids and may be of importance in the homeostasis of fatty acid levels [18]. Overexpression of Cytochrome P450 has been studied previously and has shown to inhibit microsomal Ca^{+2} ATPase, and has also been known to release reactive oxygen species which alter calcium homeostasis[19].

Much like the Acyl-Coa (transcript of the ACSL6 gene) the CVP2E1 gene was down regulated almost to the same degree. As Acyl-CoA is a coenzyme which regulates the metabolism of fatty acids, it remains to be determined whether its regulation could play a role in the regulation of cytochrome P450. It is important to elucidate that these changes in the genes that regulate fatty acid metabolism did not show regulatory significance in other studies using

shorter training time points [4]. This may well be a long term metabolomic change that takes place after long training periods of exercise endurance.

In the study comparing gene expression between fully trained animals and those after an strenuous acute exercise had completely different gene regulators that were not seen from mid-training to fully trained animals (fig 5).

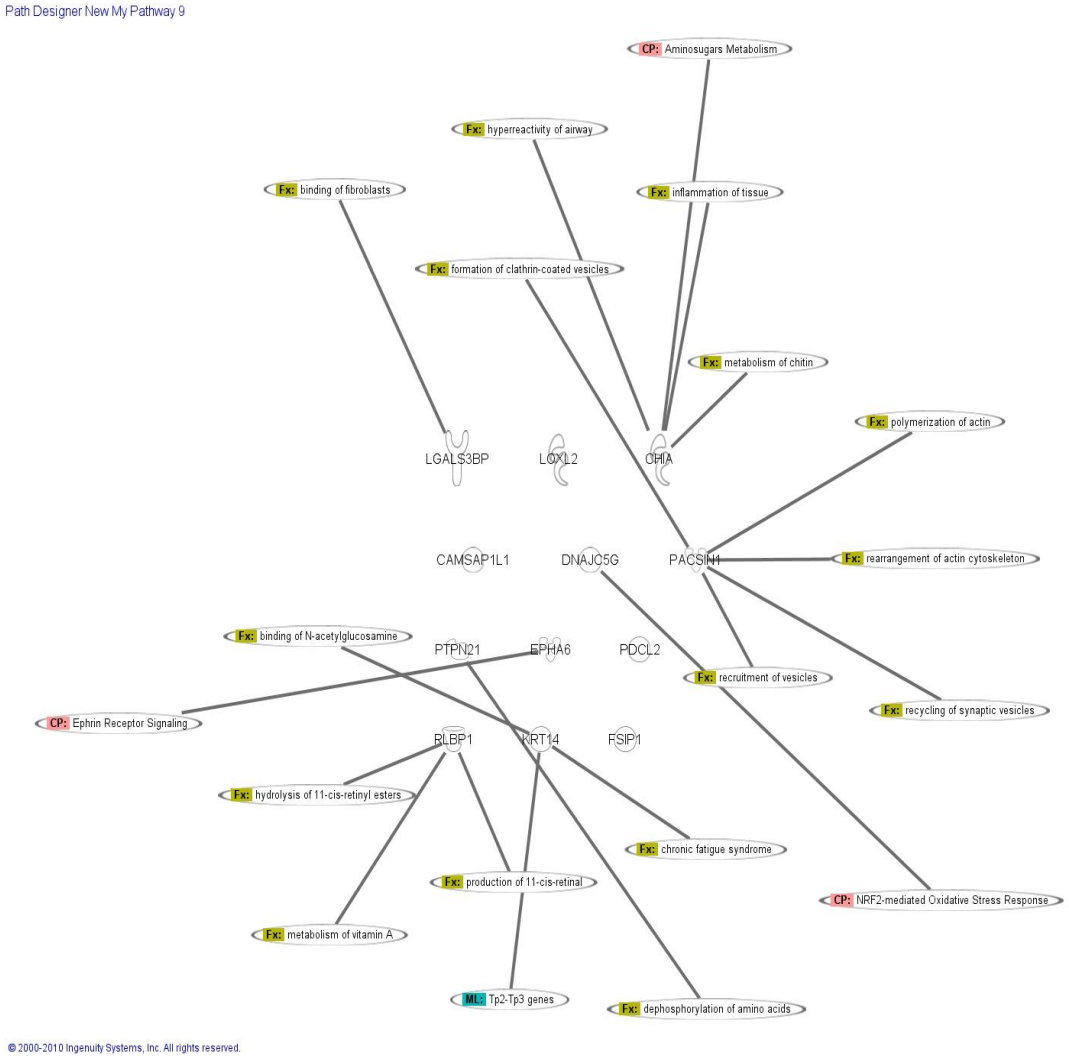

Fig 5. Pathway for interaction of the 13 mapped genes found to be differentially expressed between Tp3-Tp4 as well as their known functions.

One such transcript was the lysyl oxidase transcript, which may have a long term effect over cardiovascular remodeling to permit increased blood flow to tissues and organs. This transcript is a chemokine involved in the migration of varied cell types and generates chemotactic responses of the cell to protein oxidation [20]. Lysyl oxidase transcript has recently been known to interact with PDGFR which is a factor known to act over cell proliferation, cellular differentiation, cell growth, and development. PDGFR also exhibits significant affinity for subsets of tyrosine kinase, which was another transcript shown to be up-regulated at Tp4 [21]. However, the up-regulation of the lysyl oxidase transcript in this study could be construed as regulation of remodeling taking place in the muscle fibers in response to the acute exercise.

Protein tyrosine phosphatase is a major negative regulator of insulin and leptin sensitivity, this transcript enhances leptin action in neurons and regulates the metabolism via the nervous system [22, 23]. It is widely expressed in the skeletal muscle and its deficiency has been known to cause insulin hypersensitivity while also diminishing fat stores in the muscle [24]. These dogs have shown to have hypersensitivity to insulin, so the effects of this gene over the metabolism of insulin may be regulated by other transcripts in similar pathways. However, the up-regulation of this gene at Tp4 could be more a result of energy requirements and serve as an activator of certain nervous system responses to chronic fatigue syndrome.

The KRT14 gene (keratin 14) was down regulated between Tp2-Tp3 yet up-regulated between Tp3-Tp4. This transcript has been related to the chronic fatigue syndrome. Though CFS is more closely related to illness, stress factors caused by the acute strenuous race these dogs performed could account for the up-regulation of this gene at the last time point. Stress response is also found to be a component regulated by the HSP40 gene (heat shock protein). This protein regulates the ATPase activity involving ATP binding through the HSP70 transcript. Overexpression of HSP40 stimulates HSP70 by enhancing the rate of ATP hydrolysis, and

increasing the rate of nucleotide exchange [25, 26]. Other studies have shown that HSPs work in cardio protection during acute or prolonged exercise, a particular cytoprotective role against cardiac protein damaging stresses has been attributed to HSP70. However, studies revealed that exercise in cold environments could provide protection against same cardiac insults without over expression of HSPs [27]. Though we can't fully explain the effect of the HSP40 transcript to be down-regulated, these Alaskan sled dogs undergo serious metabolic changes that activate alternative energy source expenditure for them to withstand and endure the strenuous exercise, and they are subjected to environments of extreme cold temperatures. This result in HSP regulation in these athletes would be interesting to study further.

When analyzing gene expression between Tp1-Tp3, thirty down-regulated genes and five up-regulated genes were mapped to specific pathways and functions. The ubiquinol-cytochrome-c reductase gene which is involved in regulating reactive oxygen species and oxidative phosphorylation reduction was up-regulated, while the GAPDH gene also involved in oxidation reduction was down-regulated. The GAPDH gene is also known to be involved in phosphorylation, glycolysis and glucose metabolic processes. Though it would be hard to explain why this specific gene was down-regulated between Tp1 and Tp3, looking more in depth into the up-regulated genes we find that there is certain control up-stream that regulates the same pathways such as glycolysis and gluconeogenesis. The different genes negatively or positively expressed seem to activate a downward cascade that maintains perfect homeostasis of the metabolism in question.

The main finding between the untrained and fully trained dogs was a gradient of down-regulated genes (table 2) involved in energy metabolism, such as ATP catabolic processes, actin-dependant ATPase activity, and lipid metabolic process. This in our opinion can be explained by the evidence that the control of these specific pathways in these dogs have evolved to generate a

high energy charge while maintaining energy efficiency in the muscular tissue. Ten genes involved in energy production, specifically generation of energy such as the ubiquinol-cytochrome-c reductase, cytochrome-b5 reductase, and those genes regulated by TGSB1 were all found to be up-regulated. Processes such as detection of oxygen, generation of precursor metabolites and energy, superoxide metabolic process, insulin secretion, glucose homeostasis, cell development, oxidation reduction are all regulated by these genes and their metabolic pathways.

5. CONCLUSION

The findings of this study further confirm the metabolic changes that take place in Alaskan sled dogs to enable them to perform exercise with efficiency like no other species. Conditioning and exercise stimulates a complex set of molecular reactions for remodeling of a variety of cellular processes in the muscle, but changes observed may often be counterintuitive to the physiological demands. At gene regulation and transcriptional levels alteration of specific biological pathways allow the athlete to adapt and perform with little to no fatigue. These alterations may be facilitated through genetic endowment in these athletes, but it is clear that different stages of training and intensity of exercises create unique signatures at transcriptional levels. These signatures may be studied and discovered through gene expression analysis. Other studies show different expression patterns of altered gene regulation across time points of exercise, but this study demonstrated that short bouts of exercise, training and acute exercise performance all have very distinct regulatory patterns that alter specific biological pathways to ensure adaptation to the conditions of training.

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